Effect of guanylate cyclase-C activity on energy and glucose homeostasis

Denovan P. Begg[1,2], Kris A. Steinbrecher[3], Joram D. Mul[1], Adam P. Chambers[1], Rohit Kohli[3], April Haller[1], Mitchell B. Cohen[3], Stephen C. Woods[1] and Randy J. Seeley[1]

[1] Metabolic Diseases Institute, University of Cincinnati, Cincinnati, OH, 45237

[2] School of Psychology, University of New South Wales, UNSW Sydney, NSW, Australia, 2052

[3] Division of Gastroenterology, Hepatology, and Nutrition, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45220

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Corresponding Author

Denovan P. Begg

School of Psychology,

University of New South Wales,

UNSW Sydney, NSW, Australia, 2052

Ph: +61-2-9385-2441

Fax: +61-2-9385-3641

Abstract

Uroguanylin is a gastrointestinal hormone primarily involved in fluid and electrolyte handling. It has recently been reported that prouroguanylin, secreted postprandially, is converted to uroguanylin in the brain and activates the receptor guanylate cyclase-C (GC-C) to reduce food intake and prevent obesity. Here, we tested CNS administration of two GC-C agonists and found no significant reduction of food intake. We also carefully phenotyped mice lacking the GC-C receptor and found them to have normal body weight, adiposity and glucose tolerance. Interestingly, uroguanylin knockout mice had a small but significant increase in body weight and adiposity that was accompanied by glucose intolerance. Our data indicate that the modest effects of uroguanylin on energy and glucose homeostasis are not centrally mediated by central GC-C receptors.

Introduction

Prouroguanylin is a peptide secreted primarily from the small intestine and is cleaved to the active hormone uroguanylin at the site of action (1). Uroguanylin and its transmembrane receptor guanylate cyclase-C (GC-C or *GUCY2C*) are predominantly found in intestinal epithelial cells and function to regulate intracellular cyclic guanosine monophosphate (cGMP) production (2). The GC-C receptor is also bound by the related intestinal hormone guanylin and bacterial heat stable enterotoxins (STs) (3; 4). Ligand-induced activation of GC-C regulates electrolyte and fluid secretion into the intestinal lumen (5). Further, this receptor regulates additional processes in the healthy and diseased gut including epithelial cell proliferation (6), colonic inflammation and infection (7-9), and sodium homeostasis (10; 11).

In addition to the secretion of prouroguanylin into the gut lumen, prouroguanylin is released into the circulation and targets multiple tissues including the kidney and brain (10; 12). Notably, uroguanylin regulates electrolyte secretion in the kidney nephron via both GC-C-dependent and GC-C-independent mechanisms (13; 14). Valentino et al. recently reported that intestinal prouroguanylin, released postprandially, may be a satiation factor that reduces feeding behavior in mice by acting at GC-C receptors in the hypothalamus (15).

The hypothesis that uroguanylin and GC-C are involved in the normal regulation of energy intake has tangential support from invertebrate studies. In both *Drosophila* (16) and *C. elegans* (17), cGMP signaling is an important factor influencing feeding behavior.

In addition, other central cGMP activators, including the natriuretic peptides and nitric oxide, also alter energy balance by suppressing food intake (18; 19).

Because of the potential importance of a cGMP-based CNS circuit regulating food intake and energy homeostasis, we sought to determine the effects of CNS administration as well as loss of function of both the receptor and uroguanylin on energy balance regulation. In a series of experiments we observed no effect on food intake or body weight following administration of uroguanylin, nor of the GC-C agonist ST, into the brain. Further, there were no differences of body weight; adiposity or glucose tolerance between GC-C-deficient mice and their littermate controls. However, uroguanylin-deficient mice had increased weight gain, adiposity and glucose intolerance. While our data do not support a role for GC-C and uroguanylin in the hypothalamic control of food intake, uroguanylin does appear to influence energy and glucose homeostasis potentially via mechanisms that are independent of the GC-C receptor.

Methods

Animals and Housing

Adult male Long–Evans rats (250–300g; Harlan, IN) were housed in individual tub cages. GC-C(-/-&+/+) and UGN(-/-&+/+) mice were bred from heterozygous pairs on a >10 generation C57Bl/6 background and maintained 2-3 per cage until the final 4-weeks of the experiment when they were separated to perform a mixed meal tolerance. All animals were maintained on a 12:12-h light:dark cycle in the AAALAC-accredited animal facilities of the Metabolic Disease Institute of the University of Cincinnati. Except where specified, animals had *ad libitum* access to water and pelleted diet. Rats

were maintained on a low-fat chow diet (Harlan Teklad Rat Chow, WI, 3.1kcal/g, percentage of energy from fat:17%, carbohydrate:58%, and protein:25%). Mice were maintained on the low-fat chow diet until 10-weeks of age and were then provided with high-fat diet (HFD, n=40, Open Source Diets, NJ. 4.54kcal/g, percentage of energy from fat:40%, carbohydrate:45%, and protein:15%). All protocols were approved by the University of Cincinnati Animal Care and Use Committee.

Third-ventricular cannulation (I3VT)

Surgery was performed using sterile techniques, as previously described (20). Briefly, rats (n=22) were anesthetized using ketamine (70mg/kg) and xylazine (6mg/kg) intraperitoneally, shaved and positioned in a stereotaxic instrument (Kopf Instruments, CA). A stainless-steel cannula (Plastics One, VA) was positioned 7.5mm ventral to the dura, on the midline 2.2mm posterior to bregma, the cannula was fixed to the skull with dental acrylic anchored by screws. Placement of the cannula was confirmed behaviorally by injecting 10ng of angiotensin II (American Peptides, CA) in 1µL normal saline through the cannula. Rats that consumed >5mL of water within 30min were considered to have a viable cannula; 2 rats were excluded.

I3VT Injections and food intake

Animals were handled and weighed daily. Rats had chow removed for the final 4 h of the light phase on both the day prior to and the day of injections. Injections were performed in a repeated-measures counter-balanced manner with each injection made at least 4 days apart, so that each animal received 2-uL injections of uroguanylin (5,10,25, or 50µg, Sigma-Aldrich, MO) and a saline control, a dose response was performed due to no

published data relating to central uroguanylin administration. Animals were then administered the GC-C activator ST (1μg, Sigma-Aldrich), exendin-4 (1μg) as a positive control or a saline control, also in a repeated-measures counter-balanced manner. Injections were given 30 min before the onset of dark. Food hoppers were returned at the onset of dark and intake was assessed 1,2,4 and 24h after food return. Body weight was measured prior to injection and after 24h.

Diet-induced obesity in GC-C and UGN mice

At 10 weeks of age, male mice (n=10-12/genotype) were provided with a HFD for 16 weeks. After 12 weeks on the HFD mice were separated into individual cages to measure food intake. Mixed meal tolerance was performed after 14 weeks on the HFD. Body composition analysis was performed using NMR prior to and after 16 weeks on the HFD.

NMR body composition analysis

Body composition (fat mass and lean mass) was assessed using nuclear magnetic resonance (NMR) in conscious mice (Echo NMR, TX).

Mixed-meal tolerance test

Following a 4-h fast, mice were gavaged with a mixed-meal, Ensure (200μL/mouse). Blood glucose was assessed at baseline,15,30,45,60,120 and 240min (Accu-Chec; Roche Diagnostics, IN). Insulin was assessed at baseline and 15min by ELISA (Crystal Chem, IL).

cGMP activity in hypothalamic tissue

cGMP activity was assessed by EIA using hypothalamic tissue dissected 30 min after I3VT of either 10μg uroguanylin, 1μg ST, or vehicle. The hypothalamic block was dissected between the optic chiasm and the dorsal edge of the fornix tissue was homogenized in 5%TCA, centrifuged and extracted with water-saturated ether. Samples were acetylated and then assayed according to the manufacturer's directions (Cayman Chemical, MI).

Gene expression

Gene expression was assessed using RT-PCR similar to previous reports in rat hypothalamic tissue. Briefly, RNA was extracted from tissue using tri-reagent, assessed for quality using a spectrophotometer and converted to cDNA. Expression of GC-C and uroguanylin were determined using gene-specific probes in accordance with manufacturer's instructions (Applied Biosystems, CA).

Statistical Analyses

Data were analyzed using repeated-measures ANOVA, one-way ANOVA or repeated-measures t-tests as appropriate. Post hoc Tukey tests were performed where significant interactions were observed in ANOVAs. Significance was accepted at $p<0.05$ with data reported as mean±SEM.

Results

Third-ventricular (I3VT) uroguanylin and GC-C agonism does not inhibit food intake or reduce body weight. I3VT uroguanylin, at increasing doses (0-50μg), did not alter food intake in rats after 1,2,4 or 24h following infusion relative to vehicle, saline (Figure-

1A). Similarly, 24h body weight was not affected by I3VT uroguanylin (Figure-1B). To determine if I3VT uroguanylin has a more transient effect, food intake was assessed 15 and 30min following I3VT uroguanylin (25μg) in an additional experiment but, again, no difference was observed between uroguanylin and saline (Figure-1C). I3VT administration of the GC-C agonist ST (1μg) also had no effect on food intake whereas I3VT administration of exendin-4 (1μg), a GLP-1 agonist, produced a marked reduction of food intake at 1, 2, 4 and 24h in the same animals ($p<0.05$; Figure-1D). Body weight was reduced only following exendin-4 infusion ($p<0.05$; Figure-1E).

The GC-C receptor is present in the hypothalamus and cGMP production is increased in response to I3VT uroguanylin and ST. A failure to alter food intake could result from the administered compound not activating hypothalamic GC-C receptors. Initially, we used real-time RT-PCR to confirm the presence of GC-C receptor mRNA in hypothalamic tissue (no detectable uroguanylin mRNA was found) (Figure-1F). I3VT administration of uroguanylin (10μg) or ST (1μg) increased cGMP production in hypothalamic tissue (Figure-1G), demonstrating that the administered ligands were biologically active and capable of activating GC-C receptors.

Diet-induced obesity develops normally in GC-C-/- mice but is modestly increased in UGN -/- mice. Relative to wild-type littermate controls, GC-C deficient (GC-C-/-) and uroguanylin deficient (UGN-/-) mice displayed no change of body weight (Figure-2A), fat mass (Figure-2B) or lean mass at 8 weeks of age when maintained on low-fat chow (Figure-2C). When maintained on high-fat diet (HFD), GC-C-/- mice had equivalent body weight gain as their littermates (GC-C+/+) (Figure-2D). UGN-/- mice had greater weight gain than UGN+/+ mice ($p<0.05$; Figure-2E), indicating an increased propensity

to develop diet-induced obesity. Adipose tissue was increased comparably in GC-C-/- and GC-C+/+ mice after 16 weeks on a HFD. In contrast UGN+/+ had significantly less adipose tissue compared with UGN-/- mice ($p<0.05$; Figure-2F). Lean mass was not affected by GC-C or UGN genotype (Figure-2G). Mean daily food intake was not effected by GC-C genotype (GC-C+/+ 3.26±0.32g/day; GC-C-/- 3.23±0.24g/day) but was moderately increased in UGN -/- mice (3.37±0.21g/day) relative to their controls (2.80±0.18g/day; $p<0.05$).

GC-C-/- mice have normal mixed-meal tolerance whereas UGN-/- mice have significant insulin resistance. Fasting glucose was not different in GC-C-/- or UGN-/- mice relative to their littermate controls (Figure-3A). Following an intragastric mixed-meal gavage, blood glucose excursions were comparable between GC-C-/- and GC-C+/+ mice (Figure-3B). UGN -/- mice had a significantly greater glucose excursion than UGN+/+ mice ($p<0.05$; Figure-3C). Fasting insulin levels were unaffected by GC-C or UGN genotype (Figure-3D).

Discussion

In contrast to a previous report in the mouse (15), acute I3VT administration of uroguanylin or the GC-C agonist and enterotoxin, ST, elicited no changes of food intake or body weight in the rat. Despite this, we determined that GC-C mRNA is present in the hypothalamus, as has previously been reported (15), and that hypothalamic tissue is sensitive to I3VT-administered uroguanylin and GC-C, at least with regard to cGMP production. Together these data indicate that whereas GC-C is present and can be

activated in the hypothalamus, its activation is unrelated to the short-term regulation of food intake or body weight.

Additionally, we observed no effect of GC-C knockout on any measure of metabolic status on either a chow diet or during maintenance on a HFD and development of diet-induced obesity, with GC-C-/- mice maintaining body weight, body composition and food intake at the same levels as occurred in GC-C+/+ mice. This is in contrast to a previous report where GC-C knockout mice were found to be prone to diet-induced obesity (15). Both glucose tolerance and plasma insulin levels were also unaffected by GC-C genotype in the present study.

When maintained on a chow diet, uroguanylin-deficient mice had similar body weight and composition as their wildtype littermates. However, when fed a HFD, mice lacking uroguanylin had a modest increase in body weight and body fat that was the result of a modest hyperphagia relative to their wild-type littermates. When challenged with an oral mixed-meal, uroguanylin-deficient mice also displayed reduced glucose tolerance, with greater increases in blood glucose that were sustained for a longer period. This was coupled with elevated post-prandial plasma insulin, as would be predicted by the increased adiposity of the uroguanylin-deficient mice.

Interactions between diet and intestinal microflora impact energy balance and intestinal bacterial can increase the efficiency of caloric harvesting and the level of fat deposition in adipose and liver (21). The outgrowth of obesity-prone commensals may be especially problematic when coupled with intestinal barrier dysfunction, as the enhanced release of bacterial components into the portal circulation drives the development of insulin

resistance, fatty liver disease, and the low grade systemic inflammation associated with obesity (22). Importantly, GC-C loss-of-function is associated with gut microflora dysbiosis and intestinal barrier dysfunction (7; 23; 24). Further work will be necessary to determine the potentially differential responses of GC-C-/- versus UGN-/- mice to high fat diet with respect to gut commensal outgrowth and release of bacterial components into the circulation. It remains possible that uroguanylin impacts body weight and insulin sensitivity via multiple mechanisms.

The disparities between our data and Valentino and colleagues report (15) are not easily explained, in part methodological differences may have contributed to the conflicting data. For example, we allowed the mice to mature on low-fat chow prior to high-fat diet feeding, additionally our report focused only on only male animals as opposed to a mixture of males and females, and finally, our GC-C agonist studies were performed in rats, not mice. Despite these methodological variations, based on the differences we have observed between GC-C-/- and UGN-/- animals it appears that the effects of uroguanylin of energy balance are independent of the GC-C receptor.

Collectively, our data suggest that uroguanylin deficiency produces modest effects on both energy and glucose homeostasis. However, the data further indicate that these effects are not mediated by the GC-C receptor. In the limited evaluation of humans with either loss or gain of GC-C receptor function, either body weight has not been reported or no difference was observed (5; 25). Collectively, our data suggest that the relationship between uroguanylin and energy homeostasis is more complicated than has been previously suggested (15) and may not be a viable target for therapeutic intervention.

Author contributions

D.P.B., K.A.S., R.J.S. designed the experiments. D.P.B., K.A.S., J.D.M., A P.C., R.K. and A.H. collected and analyzed the data. K.A.S. and M.B.C. provided essential reagents and analytical tools. D.P.B., K.A.S., S.C.W. and R.J.S. wrote the manuscript. All authors reviewed and edited the manuscript. D.P.B. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Disclosure statement

The authors report no conflict of interest. R.J.S. has consultancies, research support or is a paid speaker with the following companies: Ethicon Endo-Surgery/Johnson & Johnson, Novo Nordisk, Merck, Novartis, Angiochem, Zafgen, Takeda, Ablaris, Pfizer, Eli Lilly, Zealand Pharma.

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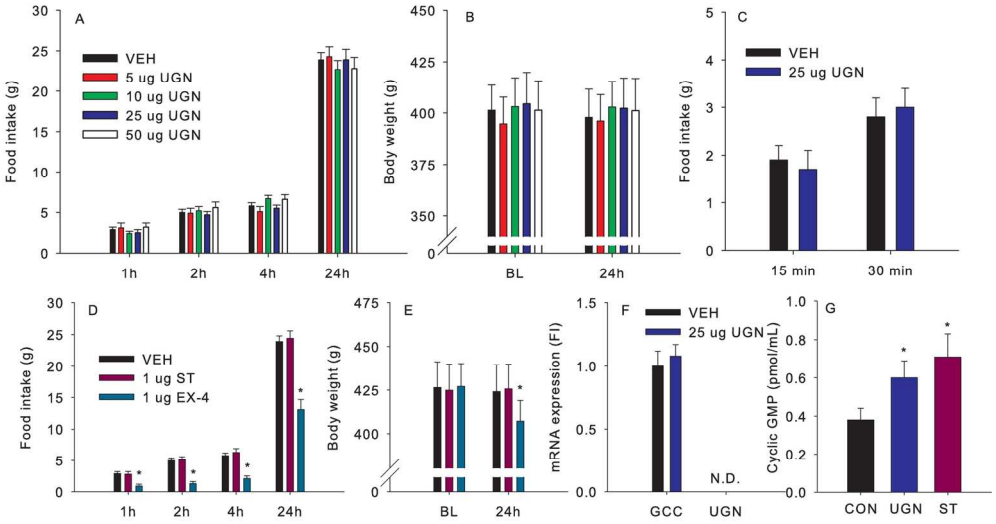
Figure Legends

Figure 1. ***I3VT uroguanylin (UGN) and bacterial the enterotoxin ST do not inhibit food intake or reduce body weight.*** (A) No reduction of food intake was observed 1, 2, 4 or 24 hours after increasing doses of UGN. (B) Body weight at 24 hours was also unaffected relative to baseline (BL). (C) Additionally, there was no short-term impact of UGN on food intake at 15 or 30 min. The GC-C agonist, ST, also produced no change in (D) food intake or (E) body weight despite the positive control, exendin-4 (EX-4), reducing both. (F) Transcripts for GC-C, but not UGN, were present in hypothalamic tissue, and (G) cGMP was produced in response to I3VT UGN or ST indicating presence and activity of the receptor in the hypothalamus independent of energy balance.

Figure 2. ***Diet-induced obesity develops normally in GC-C deficient mice but is modestly increased in UGN deficient mice.*** At eight weeks GC-C and UGN deficient mice maintained on a low-fat chow diet had no body weight (A), fat mass (B), or lean mass (C) differences relative to wild type controls. When maintained on high-fat diet GC-C deficient mice had similar weight gain to wild-type littermates over 16 weeks (D). UGN deficient mice on high-fat diet had increased weight gain relative to wild-type littermates from week 11 (E). The increased body weight of UGN deficient mice was reflected in an increased fat mass after 16 weeks on high-fat diet (F), whereas lean mass was unaffected by genotype (G).

Figure 3. ***GC-C deficient mice have normal tolerance to a mixed-meal whereas UGN deficient mice have significant insulin resistance.*** Fasting blood glucose was not altered in either genotype (A). Response to a mixed meal gavage was not different between GC-

C deficient and wildtype littermates (B). In contrast UGN deficient mice had greater glucose excursions than wildtype mice following a mixed meal gavage (C). Baseline insulin levels were not altered based on genotype; 15 minutes after the mixed meal gavage, UGN deficient mice had elevated insulin compared with wildtype littermates (D).



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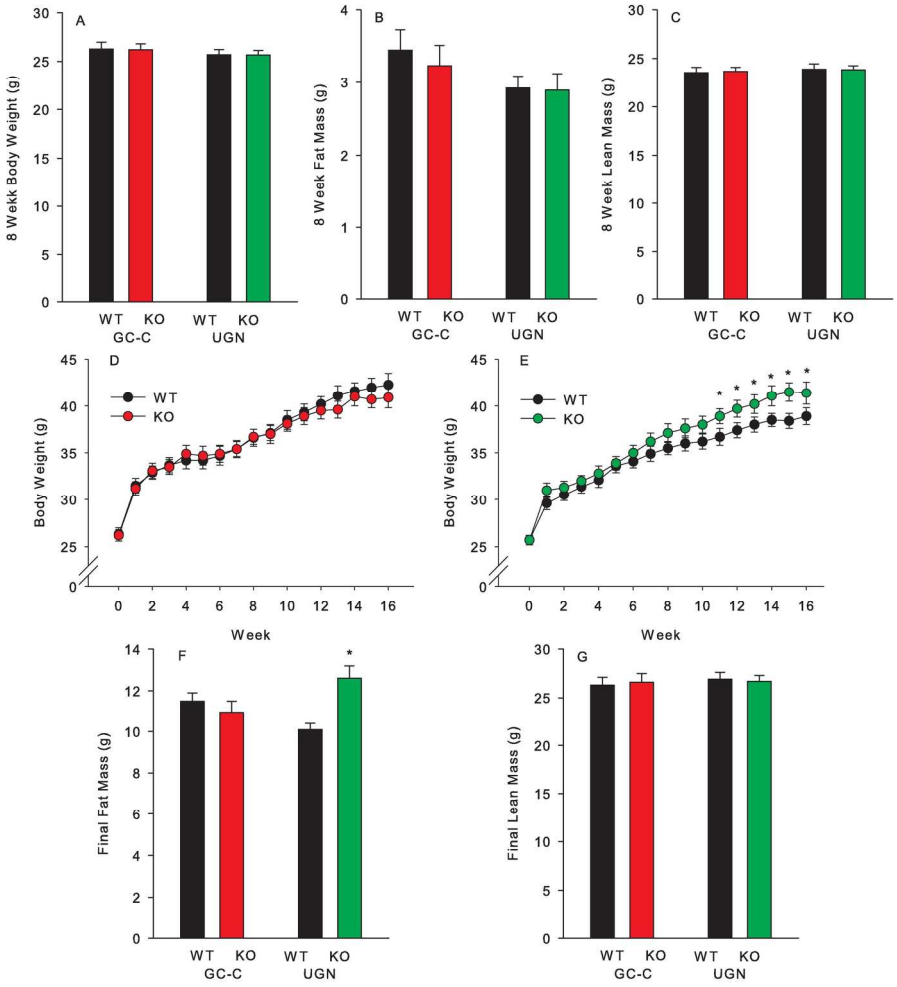

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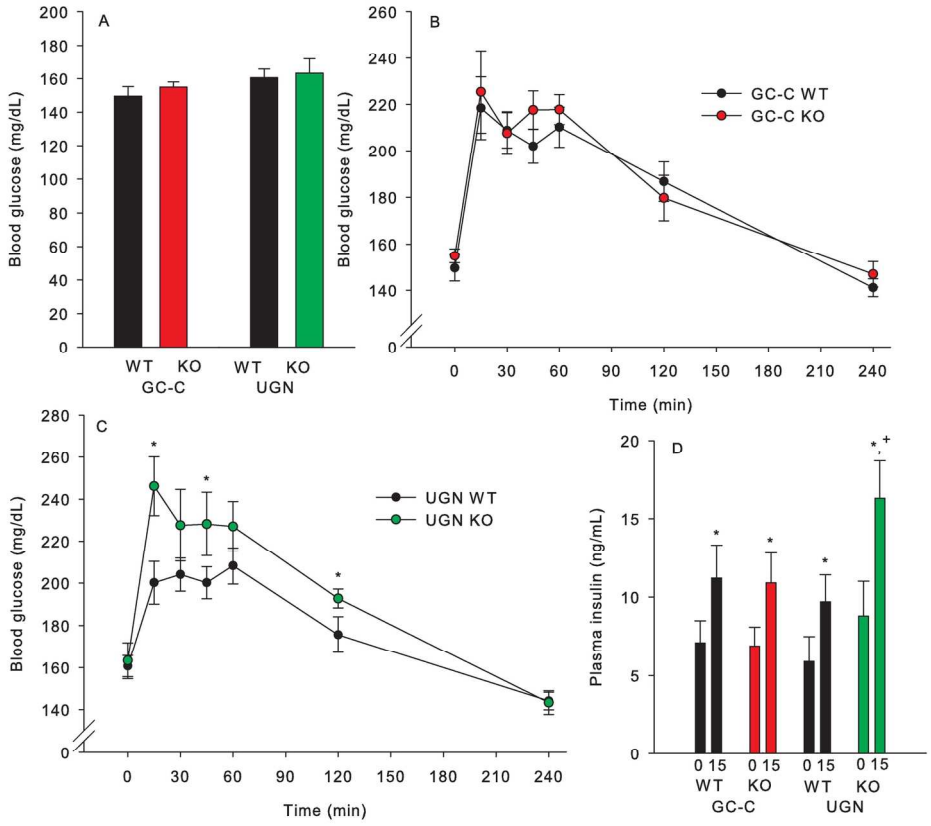

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